Enhancement of Merocyanine 540-mediated Phototherapy by Salicylate

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ABSTRACT

Merocyanine 540 (MC540) is a photosensitizing dye of potential use in the purging of cancer cells from autologous bone marrow explants. Treatment of marrow with MC540, followed by illumination with visible light, selectively kills neoplastic cells while sparing a sufficient number of stem cells to allow marrow engraftment. The photodynamic action of MC540 is thought to be mediated by reactive oxygen species, particularly singlet oxygen. We have previously shown that salicylic acid (SA) scavenges MC540-generated singlet oxygen. In this work, we sought to abrogate MC540-mediated cell killing of murine L1210 and human K562 leukemia cells with salicylate. Paradoxically, the presence of salicylate during illumination in the presence of MC540 appreciably enhanced cell killing. Enhancement was dependent on salicylate concentration in the range 0.1 to 10 μM, with 1.0 μM SA potentiating the MC540-mediated reduction in survival of L1210 and K562 cells by factors of 2.7 and 1.9, respectively. Neither preincubation with SA followed by washing prior to illumination nor addition of SA following illumination altered MC540-mediated cell killing, indicating that potentiation was dependent on the presence of SA during illumination. Illumination in the presence of salicylate alone did not diminish cell viability. In addition to SA, a number of structurally related compounds including dihydroxybenzoic acids, aspirin, and sodium benzoate also enhanced MC540-mediated cell killing. Potentiation of leukemic cell killing by salicylate could provide a basis for enhancing the clinical efficacy of MC540-mediated phototherapy.

INTRODUCTION

PDT3 is a modality for the treatment of neoplastic disease involving the introduction of a photosensitizing dye which selectively localizes in or is preferentially retained by tumor cells, followed by irradiation with visible light. Irradiation of the sensitiser initiates photothermal processes leading to the destruction or modification of the target tissue. Early clinical trials of porphyrin-based PDT have shown promise (1, 2). The mechanisms of photodynamic action in most cases involve molecular oxygen (3–5)), with the effects of PDT being mediated by reactive oxygen species such as singlet oxygen (the 1/2g excited state, 1O2), hydroxyl radical, and superoxide. Therefore PDT represents a selectively induced oxidative stress.

MC540 is a lipophilic photosensitizer with potential clinical applications in the ex vivo purging of tumor cells from autologous bone marrow explants (6–9) and sterilization of enveloped viral contaminants in blood products (10, 11). In the presence of serum, MC540 shows a marked selectivity for transformed cell types (including leukemia, lymphoma, and neuroblastoma cells) relative to normal hematopoietic cells (6–9, 12). The efficacy of MC540/PDT has been established in a number of preclinical models of marrow-purging regimens. Singlet oxygen is the primary reactive oxygen species produced during irradiation of MC540 in model systems (13–15), and MC540-mediated killing of murine leukemia L1210 cells is oxygen dependent (16). However, the basis of selectivity and the precise mechanisms of photodynamic action in cellular systems are not known.

To further establish the role of 1O2 in MC540/PDT, we sought to modulate cell killing in vitro by the addition of SA. SA reacts readily with hydroxyl radical (17, 18), and we have previously shown that SA is capable of scavenging 1O2 produced during irradiation of MC540-treated liposomes (15). However, rather than conferring protection, as might be anticipated for a compound which traps activated oxygen species, we find that the presence of salicylate during MC540/PDT potentiates tumor cell killing. This effect could provide a basis for enhancing the efficacy of MC540-mediated PDT.

MATERIALS AND METHODS

MC540 was obtained from Sigma (St. Louis, MO) and stored as small, preweighed aliquots at –20°C until use. The day of the experiment, the dye was dissolved in 50% ethanol at a concentration of 1 mg/ml and protected from light. Cell culture-tested L-histidine and L-phenylalanine were also from Sigma. SA, sodium salicylate, and each of the isomeric DHBA were from Aldrich (Milwaukee, WI). Fetal bovine serum was obtained from Hyclone Laboratories, Inc. (Logan, UT).

K562 human leukemia (American Type Culture Collection CCL 243) and L1210 murine leukemia (American Type Culture Collection CCL 219) cells were a gift from Dr. Fritz Seiber and were maintained in RPMI/10% FBS. Some experiments were conducted with cells passed several (>3) times and suspended in α-modified Dulbecco's medium (Sigma) supplemented with 10% FBS to determine if observed effects were medium type dependent. Virtually identical results were obtained with α-medium and RPMI 1640.

For photosensitization experiments, nonadherent cells were harvested from logarithmically growing suspension cultures, pelleted, and resuspended in fresh RPMI/10% FBS. Cells were then mixed in sterile 15-ml modified polystyrene centrifuge tubes (Costar, Cambridge, MA) with an equal volume of RPMI/10% FBS containing MC540, and, if desired, SA (or other drug) to give final concentrations of 1 x 10⁶ cells/ml and 15 μg/ml MC540. Vehicle-treated (i.e., no MC540) controls (both with and without SA or other drug) as well as appropriate dark controls (vehicle- and dye-treated samples) were included in each experiment. Illumination was carried out between two parallel banks of 40-W cool-white fluorescent bulbs filtered through clear Mylar. Cell suspensions were gently swirled every 10 min to prevent settling. The fluence rate was 250 mW/cm² monitored with a calibrated photometer.

In vitro clonal assays for cell survival were performed as described previously (6, 19, 20). Serial dilutions of cells (5 x 10⁴–10⁶/ml) were suspended in RPMI/10% FBS, centrifuged at 1000 g to remove unattached cells, and then resuspended in fresh RPMI/10% FBS. Cells were then mixed in sterile 15-ml modified polystyrene centrifuge tubes (Costar, Cambridge, MA) with an equal volume of RPMI/10% FBS containing MC540, and, if desired, SA (or other drug) to give final concentrations of 1 x 10⁶ cells/ml and 15 μg/ml MC540. Vehicle-treated (i.e., no MC540) controls (both with and without SA or other drug) as well as appropriate dark controls (vehicle- and dye-treated samples) were included in each experiment. Illumination was carried out between two parallel banks of 40-W cool-white fluorescent bulbs filtered through clear Mylar. Cell suspensions were gently swirled every 10 min to prevent settling. The fluence rate was approximately 2.5 mW/cm², as measured with a Yellow Springs model 65A radiometer (Yellow Springs, OH). Following illumination the cells were washed twice in RPMI 1640 containing 5% FBS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin and then resuspended for clonal assay as described below.

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3 The abbreviations used are: PDT, photodynamic therapy; MC540, merocyanine 540; 1O2, singlet oxygen; SA, salicylic acid; DHBA, dihydroxybenzoic acid(s); FBS, fetal bovine serum; MC540/PDT, merocyanine 540-mediated phototherapy; HPLC, high performance liquid chromatography; RPMI/10% FBS, RPMI 1640 supplemented with 10% fetal bovine serum.
RESULTS

The effects of salicylate on the survival of L1210 murine leukemia cells subjected to MC540/PDT are shown in Fig. 1. Illumination of L1210 cells with visible light in the presence of MC540 and SA markedly enhanced cell killing over that observed for illumination in the presence of MC540 alone. Similar results were obtained with K562 human leukemia cells (Fig. 2), indicating that this phenomenon is not restricted to a single cell line. Salicylate enhancement was also not dependent on medium type, with experiments in RPMI 1640 and α-medium (data not shown) giving similar effects. Salicylate effects were most pronounced during the early stages of clonal reduction. Survival curves as a function of illumination time for SA-treated samples were characterized by a very short initial shoulder followed by a logarithmic reduction phase that was comparable in rate to that observed with MC540 alone (Figs. 1 and 2). Salicylate enhancement factors, calculated from the irradiation dose required to reduce cell populations to 10% survival in the presence and absence of SA, averaged 2.70 for L1210 and 1.90 for K562 cells.

Enhancement of MC540/PDT by SA was concentration dependent in the range 0.1–10 mM. Under standard irradiation conditions (see Table 1), log reductions (for at least 3 independent experiments) in clonal survival were 1.72 ± 0.46 (SE), 2.65 ± 0.17, and 3.97 ± 0.02 for L1210 cells treated in the presence of 0.1, 1.0, and 10 mM SA, respectively. Under similar conditions with MC540 alone, the log reduction was 0.83 ± 0.21, with no observable change at 0.01 mM SA. Survival of cells illuminated (or incubated in the dark) with SA in the absence of MC540 was not appreciably affected in this concentration range for either L1210 or K562 cells (data not shown). Additionally, neither 2,3-DHBA nor 2,5-DHBA, with the latter being the major reaction product formed from SA during MC540/PDT, had a significant effect on clonal survival at concentrations up to 0.1 mM. High performance liquid chromatography experiments (e.g., Refs. 15, 21, 22) have shown that the concentration of 2,5-DHBA formed under our experimental conditions is <1 μM. Thus the inherent toxicity of SA or its metabolites is not the basis for the observed salicylate enhancement during MC540-mediated cell killing.

When cells were preincubated with salicylate (1 mM SA for 1 h) and then washed prior to MC540/PDT, no enhancement of MC540-mediated cell killing was observed. Similarly, when cells were subjected to MC540/PDT without SA and then washed and resuspended for an additional hour in growth medium with or without SA, the presence of salicylate during the postillumination incubation had no effect on cell survival. Thus it appears that for potentiation of MC540-mediated cell killing, salicylate must be present during MC540/PDT.

Our initial interest in the effects of SA on MC540/PDT arose due to the ability of salicylate to trap both \textsuperscript{1}O2 (15) and hydroxyl radical.

![Fig. 1. Survival curves for L1210 cells exposed to increasing doses of light in the presence of MC540 (15 μg/ml) with (•) and without (○) the addition of 1 mM salicylic acid. Points and error bars, means ± SE for three independent experiments, with a minimum of four replicate plates counted for each data point in a given experiment. * P < 0.002; ** P < 0.05.](image)

![Fig. 2. Survival curves for K562 cells exposed to increasing doses of light in the presence of MC540 (15 μg/ml) with (•) and without (○) the addition of 1 mM salicylic acid. Points and error bars, means ± SE for three independent experiments, with a minimum of four replicate plates counted for each data point in a given experiment. * P < 0.001; ** P < 0.02.](image)

Table 1 | Enhancement of MC540/PDT by salicylate analogues | Log reductions$^a$
<table>
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<td>Compound</td>
<td>n</td>
<td>Illuminated</td>
<td>Dark</td>
<td></td>
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<tr>
<td>MC540 alone</td>
<td>8</td>
<td>0.83 ± 0.21</td>
<td>0.05 ± 0.01</td>
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<tr>
<td>SA$^b$</td>
<td>8</td>
<td>2.65 ± 0.17</td>
<td>0.06 ± 0.01</td>
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<td>2,3-DHBA$^c$</td>
<td>3</td>
<td>2.76 ± 0.13</td>
<td>0.06 ± 0.01</td>
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<tr>
<td>2,4-DHBA$^c$</td>
<td>3</td>
<td>2.36 ± 0.08</td>
<td>0.03 ± 0.02</td>
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<tr>
<td>2,5-DHBA$^d$</td>
<td>3</td>
<td>3.69 ± 0.10</td>
<td>0.07 ± 0.01</td>
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<tr>
<td>2,6-DHBA$^d$</td>
<td>3</td>
<td>2.93 ± 0.46</td>
<td>0.06 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>3,4-DHBA$^e$</td>
<td>3</td>
<td>2.10 ± 0.06</td>
<td>0.73 ± 0.19</td>
<td></td>
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<tr>
<td>Catechol$^f$</td>
<td>2</td>
<td>1.94 ± 0.40</td>
<td>0.02 ± 0.01</td>
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<tr>
<td>Sodium benzoate$^f$</td>
<td>2</td>
<td>2.43 ± 0.16</td>
<td>0.02 ± 0.01</td>
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$^a$ Log reductions following treatment of L1210 cells with MC540 (15 μg/ml) in RPMI/10% FBS containing the indicated compound at a concentration of 1 mM. Samples were illuminated at 2.5 mW/cm$^2$ or kept in foil-shielded tubes for 30 s (fluence for irradiated samples, 4.5 J/cm$^2$) and then washed twice prior to plating for clonal assay (see text). Data are means ± SE from the indicated number of experiments. Statistical significance is relative to MC540 alone.

$^b$ P < 0.01.

$^c$ P < 0.001.

$^d$ P < 0.02.

$^e$ Not significant (P > 0.05).
(17, 18, 21, 22). For this reason, we examined the biological effects of phenylalanine, a scavenger of hydroxyl radicals which does not react with $^{1}\text{O}_2$ (23), and histidine, which is reactive with both hydroxyl radical and $^{1}\text{O}_2$ (17, 24). At concentrations of 1–5 mM, neither phenylalanine nor histidine altered the survival of cells subjected to MC540/PDT.

We also examined a variety of structural analogues of SA for the ability to enhance cell killing during MC540/PDT. As shown in Table 1, all of the various dihydroxybenzoic acid isomers enhance MC540-mediated killing of L1210 cells. Potentiation with 2,5-DHBA was significantly greater than that achieved with SA ($P < 0.01$). Of the compounds tested, only catechol exhibited appreciable dark toxicity (Table 1). Preliminary experiments with $\alpha$-acetylsalicylic acid (aspirin) gave a similar enhancement (log reduction, 2.62) as that observed with SA.

**DISCUSSION**

We have observed that the addition of salicylic acid during in vitro MC540-mediated PDT causes a marked increase in leukemic cell killing. This is an enhancement of the effects of PDT, rather than a synergistic effect, since neither salicylate alone, nor pretreatment with salicylate (followed by washing prior to MC540/PDT), nor the addition of SA after MC540/PDT influences cell survival.

Salicylate enhancement was dose dependent with significant ($P < 0.01$) effects observable at concentrations ≥0.1 mM SA. We chose to work at 1 mM SA since this is comparable to the serum concentrations achieved in treatment for rheumatoid arthritis (25). Higher concentrations (e.g., 10 mM) were not appreciably cytotoxic for the brief exposure in our experiments and gave greater enhancement of cell killing during MC540/PDT but may have serious complications both in vivo and in vitro (25). Nonetheless, it is apparent that salicylate produces a large potentiation in the killing of tumor cells during MC540/PDT at physiologically acceptable concentrations.

The initial aim of this study was to use salicylate as a means to abrogate the effects of oxidative stress induced by MC540 and light. SA reacts readily with hydroxyl radical (17, 18, 21, 22) and singlet oxygen (15), with the latter being the primary reactive species produced during irradiation of MC540 (13–15). It has been proposed that the antiinflammatory effects of salicylate, which does not inhibit cyclooxygenase (26), may be due in part to its ability to scavenge reactive oxygen species (27, 28). Thus the enhancement by SA of cell killing during MC540/PDT is somewhat paradoxical.

The primary reaction products of SA with hydroxyl radical are 2,3-DHBA and 2,5-DHBA with catechol as a minor product (21, 22). Reaction of $^{1}\text{O}_2$ with SA produces primarily 2,5-DHBA as a stable product, as shown previously for MC540 in a model membrane system (15). High performance liquid chromatography analysis has shown that irradiation of MC540 under our experimental conditions in the presence of 1 mM SA will produce between 0.1 and 0.01 μM 2,5-DHBA. DHBA products alone (i.e., in the dark or during irradiation in the absence of MC540) do not affect cell survival at these concentrations. Although DHBA do potentiate MC540-mediated cell killing (Table 1), their contribution to the increase in cell killing at the concentrations formed from 1 mM SA during MC540/PDT would be negligible. Possible effects due to unknown oxidation products of SA cannot be explicitly excluded, but they seem unlikely.

Salicylate produces a wide variety of physiological effects which might be related to the observed enhancement of MC540/PDT. These include alteration of both anion and cation fluxes (29), uncoupling of oxidative phosphorylation (30–35), and induced changes in membrane physical properties (28). A number of studies have examined the effects of salicylate on tissue and mitochondrial respiration (30–35), demonstrating stimulation of glyconeogenesis and uncoupling of oxidative phosphorylation with the resultant increase in O2 consumption, depletion of ATP, and increased lactate production. The early onset of ischemic contracture during perfusion of isolated rat hearts with cardioplegic solution containing salicylate may be due to these effects. However, it has been reported that none of the DHBA isomers (i.e., Table 1) examined in this study produce the changes in respiratory function noted for salicylate above (30–32). Additionally, the salicylate-induced mitochondrial swelling and proton fluxes associated with Reye's syndrome were specifically not observed with either 2,6-DHBA or benzoate (36, 37). Thus it would appear unlikely that uncoupling of oxidative phosphorylation is the basis for the observed enhancement of MC540/PDT by salicylate and related DHBA.

That the enhancement of MC540-mediated leukemic cell killing was observed with a wide variety of isomeric dihydroxybenzoates (Table 1) argues strongly against modulation of a specific physiological process. A possible effect of a more general nature would be the alteration of plasma membrane physical properties, such as a change in the molecular order (i.e., fluidity) of the lipid bilayer. Abramson and Weissmann (28) have previously reported that concentrations of salicylate as low as 0.1 mM can alter the microviscosity of the neutrophil plasma membrane (28). Such changes could influence MC540-mediated cell killing either by affecting ion transport systems or by modulating MC540 binding. Membrane lipid organization can significantly influence MC540 binding in liposomes (38, 39), although the situation in cellular systems is less clear. When a series of cell lines with varying sensitivity to MC540/PDT were ranked according to plasma membrane microviscosity, there was little correlation between fluidity and cell survival (20). Furthermore, upon manipulation of the plasma membrane fatty acid composition in cultured L1210 cells, large changes in fluidity were attained without effect on cell killing (19). Only changes in membrane cholesterol content affected MC540 uptake or cell survival. These studies indicate that membrane fluidity per se does not determine the efficacy of MC540-mediated cell killing. However, specific changes in plasma membrane structure or organization due to intercalation of SA or related planar, anionic compounds such as DHBA may provide the basis for salicylate enhancement.

Although extensive further investigation will be necessary to elucidate the mechanism(s) by which SA potentiates killing during MC540/PDT, the use of salicylate to enhance the effects of PDT in clinical applications is an intriguing concept. The success of MC540-mediated purging of cancer cells from bone marrow explants depends on the selectivity of the dye for neoplastic (especially leukemia and lymphoma) cell types relative to normal hematopoietic stem cells (6–9, 12). Since the presence of salicylate during irradiation is required to produce an enhancement, its effects may be restricted to those cells most strongly affected by MC540/PDT, i.e., the neoplastic cell population. The effects of salicylate on normal stem cell survival during MC540/PDT must be examined to determine if a net increase in the therapeutic window can be achieved.

**REFERENCES**


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SALICYLATE ENHANCEMENT OF MC540 PHOTOTHERAPY


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